

# The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography

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**This study describes the first crystal structures of a complex between a DNA topoisomerase and a drug. We present the structures of a 24 kDa N-terminal fragment of the *Escherichia coli* DNA gyrase B protein in complexes with two different inhibitors of the ATPase activity of DNA gyrase, namely the coumarin antibiotic, novobiocin, and GR122222X, a member of the cyclothialidine family. These structures are compared with the crystal structure of the complex with an ATP analogue, adenylyl- $\beta$ - $\gamma$ -imidodiphosphate (ADPNP). The likely mechanism, by which mutant gyrase B proteins become resistant to inhibition by novobiocin are discussed in light of these comparisons. The three ligands are quite dissimilar in chemical structure and bind to the protein in very different ways, but their binding is competitive because of a small degree of overlap of their binding sites. These crystal structures consequently describe a chemically well characterized ligand binding surface and provide useful information to assist in the design of novel ligands.**

**Keywords:** antibiotic/coumarin/cyclothialidine/DNA gyrase/  
X-ray crystallography

## Introduction

DNA gyrase is a member of a family of ubiquitous ATP-dependent enzymes called the type II topoisomerases. These enzymes have been implicated in many biological processes that involve DNA, e.g. they are involved in segregation of DNA after replication, initiation of DNA replication and gene expression (reviewed in Wang, 1985; Reece and Maxwell, 1991a). The active gyrase molecule is an A<sub>2</sub>B<sub>2</sub> tetramer (Klevan and Wang, 1980) and it has been demonstrated that the A protein harbours the DNA breakage–reunion domain (Reece and Maxwell, 1991b,c) and that the B protein contains the ATP binding site (Mizuuchi *et al.*, 1978; Tamura and Gellert, 1990; Wigley *et al.*, 1991).

Whereas DNA gyrase is an essential enzyme in prokaryotes, it has not been found in eukaryotes. Hence it has been seen as an attractive target for the design of antibiotics. The coumarins (e.g. novobiocin, Figure 1) are naturally occurring compounds, isolated from certain strains of *Streptomyces*, which inhibit the ATPase activity of the B protein of DNA gyrase (Hoeksema *et al.*, 1955; Gellert *et al.*, 1976b). Novobiocin comprises an unusual sugar residue, a coumarin-derived constituent and an isopentenyl-substituted hydroxybenzoate ring. The cyclothialidines are naturally occurring cyclic peptide inhibitors (Goetschi *et al.*, 1993), of which GR122222X is a member (Figure 1), and are also inhibitors of the ATPase activity of the B subunit of DNA gyrase (Nakada *et al.*, 1994). GR122222X is composed of five amino acids (two alanines, a cysteine, a serine and a *cis*-3-hydroxyproline) coupled to a substituted resorcinol ring, 3-5-dihydroxy-2,6-dimethylbenzoate (hereafter referred to as resorcinol). A lactone ring is formed between the resorcinol and the amino acids serine and cysteine.

The precise binding site is not known for either class of inhibitor. The cyclothialidines and the coumarins both inhibit the ATPase activity of the B subunit and thus they are thought to bind at, or near to, the ATP binding site. Steady-state kinetic studies of the ATPase reaction of the intact A<sub>2</sub>B<sub>2</sub> DNA gyrase molecule suggest that the coumarins and cyclothialidines are competitive inhibitors with *K<sub>i</sub>* values of the order of 10<sup>−7</sup>–10<sup>−9</sup> M (Sugino *et al.*, 1978; Sugino and Cozzarelli, 1980; Staudenbauer and Orr, 1981; Nakada *et al.*, 1994). However, studies of the intact B protein alone (Staudenbauer and Orr, 1981; Maxwell and Gellert, 1984) and a 43 kDa fragment of the B protein (Ali *et al.*, 1993) have shown that the ATPase activity of these proteins follows non-Michaelis–Menten kinetics and consequently have raised questions about the proposition that the coumarins are simple competitive inhibitors. The binding of the coumarins to DNA gyrase is apparently much tighter than for nucleotides; the *K<sub>M</sub>* for ATP is ~0.3 mM and the *K<sub>d</sub>* for adenylyl- $\beta$ - $\gamma$ -imidodiphosphate (ADPNP) is ~3  $\mu$ M compared with 10<sup>−7</sup>–10<sup>−9</sup> M for the coumarins (Sugino *et al.*, 1978; Sugino and Cozzarelli, 1980; Staudenbauer and Orr, 1981; Tamura *et al.*, 1992). Other ATP-dependent enzymes are not inhibited by similar, low concentrations of novobiocin, and proteins from *Escherichia coli*, other than gyrB, do not bind to novobiocin–Sepharose with substantial avidity (R.J.Lewis, unpublished observations). In addition, the coumarins, cyclothialidines and ATP share little structural resemblance (Figure 1) and thus the coumarins and the cyclothialidines would not be predicted to be competitive inhibitors. Furthermore, point mutations of the B subunit that confer resistance to the coumarins all lie at the periphery of the ATP binding site (del Castillo *et al.*, 1991; Holmes and Dyall-Smith, 1991; Contreras and Maxwell, 1992; Samuels

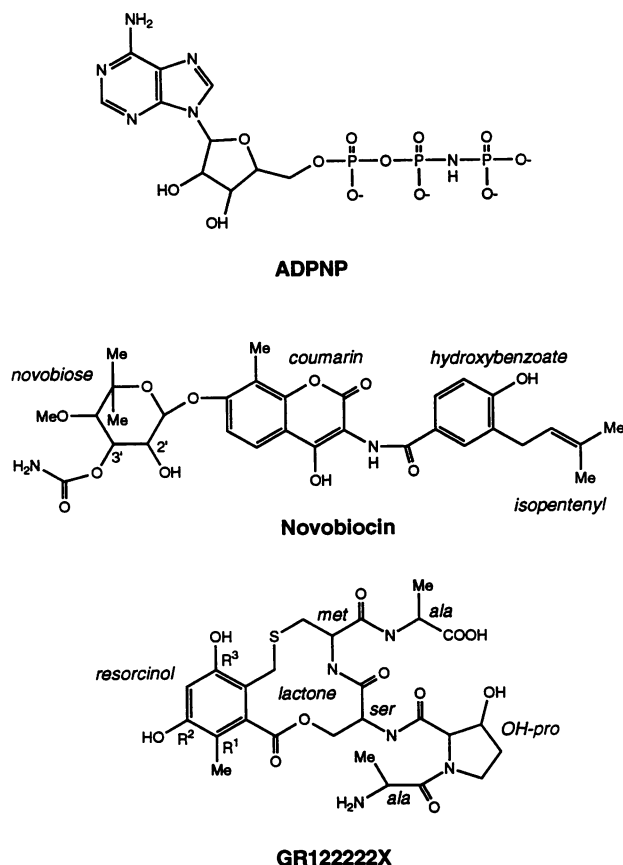


Fig. 1. The chemical structures of ADPNP, novobiocin and the cyclothialidine, GR12222X.

*et al.*, 1994). One residue in particular, Arg136, appears to play an important role in determining coumarin resistance, since a variety of mutations at this residue, from a number of prokaryotic sources, have been reported by several independent groups.

Intact gyrase B protein (gyrB) has been crystallized on two-dimensional novobiocin-linked phospholipid films and a low resolution (25 Å) model has been generated by cryo-electron microscopy and 3D image reconstruction techniques (Celia *et al.*, 1994). This model was compared with the crystal structure of a 43 kDa N-terminal fragment of the B protein (NgyrB) in a complex with ADPNP (Wigley *et al.*, 1991). The point of attachment of the protein to the novobiocin-linked phospholipid layer could be aligned with the N-terminal domain (comprising the first 220 N-terminal amino acids), suggesting that the ADPNP and novobiocin binding sites are in close proximity. All point mutations in the *gyrB* gene that confer novobiocin resistance are located in the first 220 N-terminal residues (Holmes and Dyall-Smith, 1991; del Castillo *et al.*, 1991; Contreras and Maxwell, 1992; Samuels *et al.*, 1994). A 24 kDa protein comprising the first 220 N-terminal amino acids of gyrB has been shown to bind to a novobiocin-Sepharose affinity column with similar avidity to gyrB, both requiring denaturing concentrations of urea to elute them (Staudenbauer and Orr, 1981; Gilbert and Maxwell, 1994). Therefore, it is likely that the 24 kDa N-terminal fragment of DNA gyrase B protein retains the entire coumarin binding site.

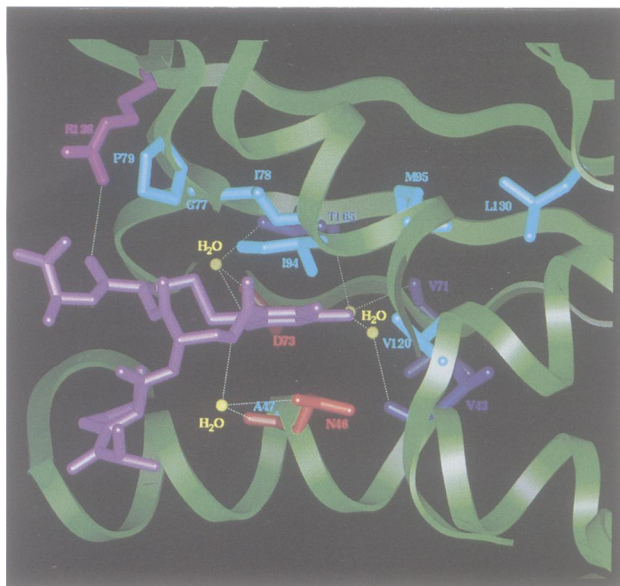
The coumarins have not enjoyed pharmaceutical success for a variety of reasons. These include poor oral absorption, low activity versus certain Gram-negative bacteria, and a tendency for coumarin-sensitive organisms readily to become novobiocin-resistant. One mechanism for this acquired coumarin resistance is believed to be due to the ability of some prokaryotes to chemically modify novobiocin, transferring the carbamoyl at position 3' of the novobiocose sugar moiety to position 2' (Kuo *et al.*, 1991). In order to determine the binding sites of the coumarins and the cyclothialidines, we have solved the crystal structures of complexes of these compounds with a 24 kDa N-terminal fragment of DNA gyrase B protein (p24). An understanding of the ligand binding site(s) should be of use in the design of improved inhibitors of DNA gyrase. To this end, we have determined crystal structures of both the p24-GR12222X complex (at 2.0 Å) and the p24-novobiocin complex (at 2.7 Å).

## Results

### Overall structure of p24 in the two inhibitor complexes

The protein component of both inhibitor complexes is structurally similar to the equivalent region of the 43 kDa B protein fragment complexed with ADPNP [NgyrB-ADPNP (Wigley *et al.*, 1991)]. Briefly, the structure comprises a compact single domain with an eight-stranded  $\beta$ -sheet which is flanked on one side by three  $\alpha$ -helices and several stretches of random coil. However, the NgyrB-ADPNP complex is dimeric and, in the crystal structure, the 2-fold axis of the dimer is coincident with a crystallographic 2-fold axis. The two p24 inhibitor complexes are both monomeric, and the absence of any pure rotational symmetry operations in space group P2<sub>1</sub>2<sub>1</sub> precludes dimer formation by crystallographic symmetry in both cases. The major differences between the three structures are confined to three regions of the protein. The first of these lies at the N-terminus, where, in the NgyrB-ADPNP complex, the N-terminal 16 residues form an 'arm' which extends away from the main body of the protein and wraps around a symmetry-related molecule to form part of the ATP binding site (Wigley *et al.*, 1991). In the p24-GR12222X complex, residues 6-15 form two additional turns of an  $\alpha$ -helix. Electron density for the N-terminal four amino acids is weak, indicating that these residues are disordered. In the case of the p24-novobiocin complex, the N-terminal 13 amino acids cannot be traced.

The second difference relates to the loop between residues 99 and 118. In the NgyrB-ADPNP complex, this loop forms a lid over the ATP binding site, Lys103 forms a salt bridge with the  $\beta$ -phosphate of ADPNP, Tyr109 forms a hydrogen bond to the N3 atom of the adenine ring and glycines 114, 117 and 119 are part of the GXXGXG motif that lies adjacent to the phosphates of ADPNP. In the p24-GR12222X complex, this loop is disordered. Indeed the hydroxyproline moiety of GR12222X is situated in such a way that Tyr109 cannot possibly attain a similar orientation to that found in NgyrB-ADPNP, because of severe steric hindrance. In the p24-novobiocin complex, this loop extends away from the rest of the protein but is stabilized in this position, in part, by interactions with a symmetry-related molecule.



**Fig. 2.** Interactions between the protein and GR12222X in the complex. Figures 2 and 3 were generated using MOLVIEWER (M.Hartshorn, personal communication).

The third difference between the structures is at a loop between residues 81 and 87. In the NgyrB–ADPNP complex, this loop is well ordered and is somewhat tethered by interactions it forms with the N-terminal arm of a symmetry-related molecule. In the p24–GR12222X complex, this loop appears to be mobile because the electron density for this loop is weak and protein atoms in this region have higher than average B-factors. In the p24–novobiocin complex, this loop is disordered and the protein chain cannot be traced.

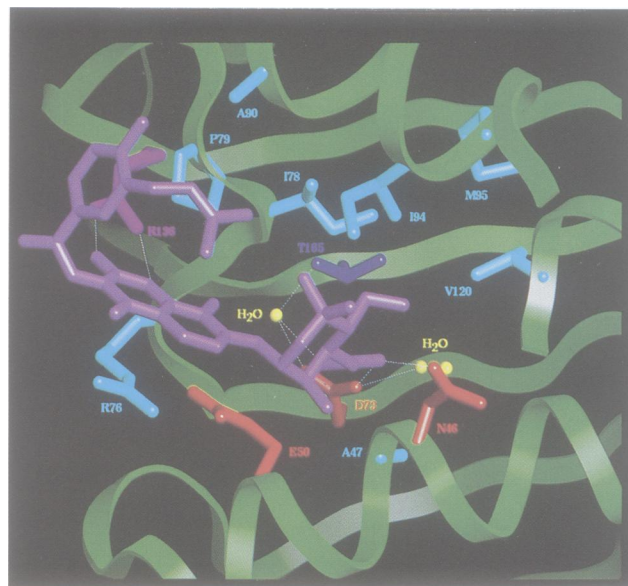
#### **Interactions between p24 and GR12222X**

The substituted resorcinol ring contributes the majority of the interactions between GR12222X and the protein. There are several hydrophobic amino acids that line the ligand binding site, e.g. valine (residues 43, 69, 71 and 120), Leu130, Ala47, Gly77, Pro79, Met95, Ile78 and Ile94 (Figure 2). The hydroxyls of the resorcinol moiety of GR12222X form hydrogen bonds with the protein (Figure 2). Replacement of the R<sup>3</sup> hydroxyl, by a hydrogen, results in a reduction in affinity of over three orders of magnitude (Goetschi *et al.*, 1993).

The hydroxyl at position R<sup>2</sup> forms water-mediated hydrogen bonds with the main chain carbonyls of Val43, Val71, Thr165 and the side chain of Asp73. Val71 is conserved amongst DNA gyrases as either valine or isoleucine, while in topoisomerase II proteins this residue is predominantly isoleucine. Further water-mediated interactions are also formed between the ester oxygen of GR12222X, the amide nitrogen of the serine of GR12222X and the main chain carbonyl and side chain of Asn46. This residue is totally conserved amongst both gyrases and type II topoisomerases, and coordinates the Mg<sup>2+</sup> ion in the ATP binding site which is necessary for ATP hydrolysis.

#### **Interactions between p24 and novobiocin**

Examination of the binding site of novobiocin reveals an extensive hydrogen bonding network, involving especially

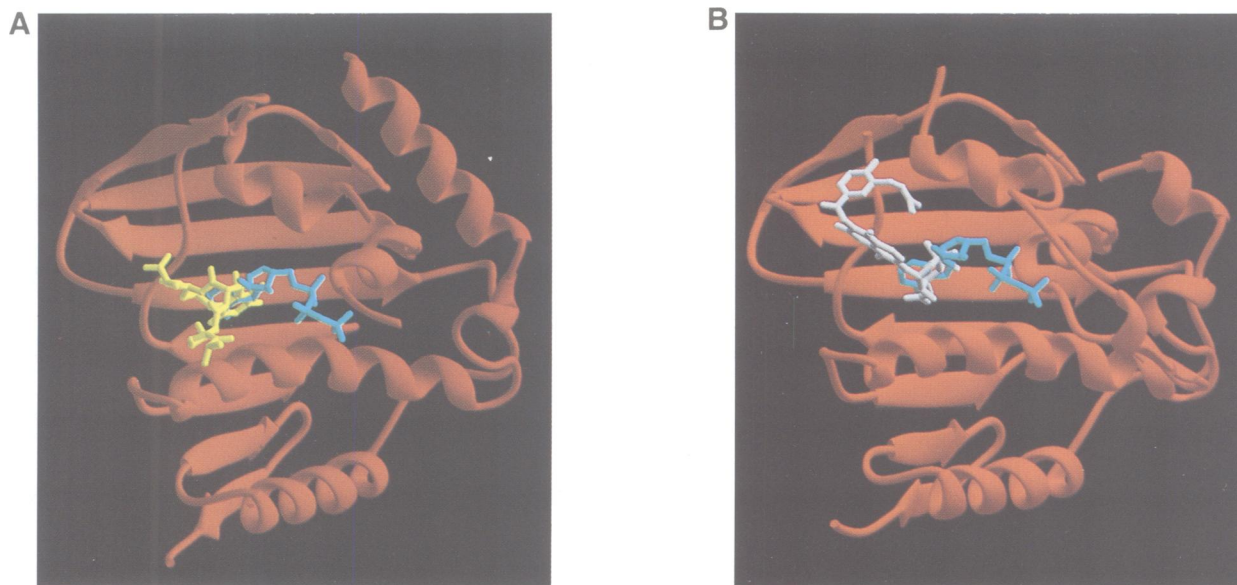


**Fig. 3.** Interactions between the protein and novobiocin in the complex.

the novobiose sugar element (Figure 3). The 3'-carbamoyl group of novobiose forms hydrogen bonds with the amide nitrogen of Ala47 and the side chain of Asp73, but also forms hydrogen bonds to three ordered water molecules. One of these water molecules forms hydrogen bonds to the main chain amide of Gly77 and to the side chains of Asp73 and Thr165. Another of these water molecules forms hydrogen bonds with the side chain of Asp73, the carbonyl oxygen of Val71 and a third water molecule. This third water molecule forms hydrogen bonds to the carbonyl oxygen of Val43. The 2'-hydroxyl of novobiose forms a hydrogen bond to the carbonyl oxygen of Asn46, and the 4'-methoxyl oxygen forms a hydrogen bond to the side chain of Asn46. Transfer of the carbamoyl from position 3' of novobiose to position 2' (isonovobiocin) has been implicated in one mechanism for resistance to novobiocin (Kuo *et al.*, 1991). Isonovobiocin has a dramatically reduced affinity for DNA gyrase. Examination of the crystal structure of the complex reveals that this is probably a consequence of steric clashes with the main chain carbonyl of Asn46. The ether oxygen that bridges the coumarin ring and the novobiose sugar forms a hydrogen bond to an ordered water molecule, which in turn forms a hydrogen bond to the side chain of Glu50. The coumarin ring forms only two hydrogen bonds, both with Arg136 (Figure 3). The ester and carbonyl oxygens of the coumarin ring are 3.2 and 2.6 Å, respectively, from the guanidium group of this amino acid.

In addition to the hydrogen bonding network, there are a number of hydrophobic contacts between the protein and novobiocin. The 5',-5'-dimethyl group of the sugar component of novobiocin is surrounded by hydrophobic amino acids. Similarly, the 4'-methoxy methyl group sits in a hydrophobic patch comprising a number of residues. The alkyl section of Arg76 lies above the plane of the coumarin ring and Pro79 lies underneath. The 3'-isopentenyl-4'-hydroxybenzoate moiety wraps around Pro79 (Figure 3). It has been shown by chemical modification of novobiocin that this group is not required for DNA





**Fig. 4.** (A) Overlap of the GR12222X (yellow) and ADPNP (pale-blue) binding sites. (B) Overlap of the novobiocin (white) and ADPNP (pale-blue) binding sites. These figures were generated using the program RIBBONS (Carson, 1991).

gyrase inhibition in supercoiling and ATPase assays (Reusser and Dolak, 1986), although the anti-bacterial efficiency of novenamine (the novobiocin sugar and the coumarin ring) was much reduced. The novobiocin sugar alone was inactive in anti-bacterial and anti-gyrase activities (Althaus *et al.*, 1988), thus it is probable that the interaction between Arg136 and the coumarin ring is vital in directing the sugar moiety to the appropriate site. It would appear that the 3'-iso-pentenyl-4'-hydroxybenzoate group of novobiocin is relatively unimportant in DNA gyrase interactions, but may influence uptake of the compound into bacterial cells.

The best two-dimensional crystals grown on the novobiocin-linked phospholipid were attached to novobiocin at the hydroxyl of the coumarin ring (Lebeau *et al.*, 1990). This hydroxyl group is solvent accessible in the crystal structure described herein.

#### Comparison of the ligand binding sites

"If an inhibitor, I, binds reversibly to the active site of an enzyme and prevents substrate, S, from binding and vice versa, I and S compete for the active site and I is said to be a competitive inhibitor" (Fersht, 1985). It is likely that ligands which compete for the same site will share the structural determinants which give rise to the binding at that particular site. However, one should bear in mind that in order for two ligands to compete for a binding site on a protein, their binding sites need only overlap in a small region. In the case of DNA gyrase, GR12222X forms a wedge or plug that, once bound to the protein, prevents the binding of ATP. The overlapping regions of the binding sites are the resorcinol ring of GR12222X and the adenine ring of ATP (Figure 4A). This region of overlap is quite small, but is sufficient to prevent the simultaneous binding of both ligands, making GR12222X a competitive inhibitor of the ATPase activity of DNA gyrase, consistent with the biochemical data presented previously (Nakada *et al.*, 1994).

Chemical modification of cyclothialidine indicates that

the minimum requirements for the inhibition of gyrase are the resorcinol and lactone rings (Goetschi *et al.*, 1993), and it is through this part of GR12222X that the majority of specific interactions (with the side chains of residues Asn46, Asp73 and Thr165) are made. These are all highly conserved amino acids which, in addition to being instrumental in the binding of GR12222X, are also important for the binding of ATP. The resorcinol ring of GR12222X forms one direct and three water-mediated hydrogen bonds with Asp73, as well as a further two water-mediated hydrogen bonds with Thr165. In the crystal structure of NgyrB-ADPNP, an ordered water molecule mediates hydrogen bonds between the adenine group of ADPNP, Asp73, Thr165 and the amide nitrogen of Gly77. Asp73 forms a direct hydrogen bond with the amino side group of ADPNP, as well as water-mediated hydrogen bonds with ADPNP and Thr165. Three ordered water molecules which mediate interactions between the ligands and the protein are conserved in the crystal structures of both complexes.

The binding sites for novobiocin and ATP also show some degree of overlap (Figure 4B). Again, the region involved is the adenine ring of ATP, but in this case the overlap is with part of the novobiocin sugar, and thus novobiocin is also a competitive inhibitor. The binding of novobiocin is mediated, in part, by ordered water molecules, as is the binding of both ATP and GR12222X. The key amino acids implicated in novobiocin binding appear to be Asn46, Asp73, Arg136 and Thr165. Arg136 forms a hydrogen bond to the carbonyl of the coumarin ring of novobiocin, but can also form a hydrogen bond with the carbonyl oxygen of Tyr5 on dimerization in the presence of ADPNP. On overlaying the crystal structures of NgyrB-ADPNP and p24-novobiocin, it can be shown that the carbonyl oxygen of the coumarin ring of novobiocin and the Tyr5 carbonyl oxygen are merely 1.2 Å apart. Asp73 and Thr165 not only have important functions in binding both ATP and GR12222X, but are also involved in the binding of novobiocin. Asp73 forms one

hydrogen bond directly with the carbamoyl of novobiocin, and two others via water molecules. The same residue forms a number of interactions with the adenine moiety of ADPNP in the NgyrB–ADPNP structure. The main chain carbonyl of Thr165 forms water-mediated hydrogen bonds with ADPNP. Novobiocin forms water-mediated hydrogen bonds with both the carbonyl and side chain of Thr165. Hydrogen bonds are also formed between a water molecule, Asp73, and the main chain amide of Gly77 of the NgyrB–ADPNP complex. In common with GR122222X, ordered water molecules which mediate the binding of novobiocin to gyrB are in similar positions to those associated with ATP binding.

Although the region of overlap with the ATP binding site is similar for both GR122222X and novobiocin, the binding site for each of the ligands is rather different (Figure 4A and B). While the binding sites for the inhibitors overlap to a greater extent with each other than for ATP, there are regions of the binding sites of both compounds which are unique. Consequently, the region of the protein which is contacted by one or other of the three ligands is quite large, and represents a large surface over which interactions can be evaluated for the design of novel inhibitors which might combine some or all of the characteristics of these different interactions.

Cyclotrialdinides, novobiocin and ATP are reported to bind competitively to gyrase. At first sight this is a surprising result, given the complete lack of structural homology between the three ligands, but in light of the crystal structures this apparent contradiction can be reconciled. Although the ligands differ considerably in their precise mode of binding, their binding sites overlap sufficiently to be mutually exclusive.

## Discussion

These crystal structures are the first topoisomerase–inhibitor complexes to be reported. In addition, these structures shed some light on the nature of the inhibition of the ATPase activity of DNA gyrase, which has been the subject of some debate (reviewed in Reece and Maxwell, 1991a; Maxwell, 1993). Sugino *et al.* (1978) suggested that the ATPase activity of DNA gyrase was inhibited in a competitive manner by novobiocin, a proposal which has been challenged more recently (Maxwell and Gellert, 1984; Ali *et al.*, 1993; Maxwell, 1993). The kinetic studies performed by Sugino *et al.* were conducted on intact ( $A_2B_2$ ) DNA gyrase, which apparently behaves in a Michaelian fashion, whereas the other studies were conducted on gyrase B protein alone (Maxwell and Gellert, 1984) or with the 43 kDa N-terminal fragment of the gyrase B protein (Ali *et al.*, 1993). These latter studies indicated a greater than first order dependence on protein concentration, i.e. non-Michaelian kinetics, and are consistent with the ATPase activity of DNA gyrase B protein(s) being dependent upon dimerization. Biophysical studies of DNA gyrase, such as electron microscopy, transient electric dichroism, neutron and dynamic light scattering, have shown that DNA gyrase is an  $A_2B_2$  tetramer in solution, even in the absence of ADPNP (Kirchhausen *et al.*, 1985; Rau *et al.*, 1987; Krueger *et al.*, 1990). However, both intact gyrase B protein and the 43 kDa N-terminal fragment of DNA gyrase B are monomeric in

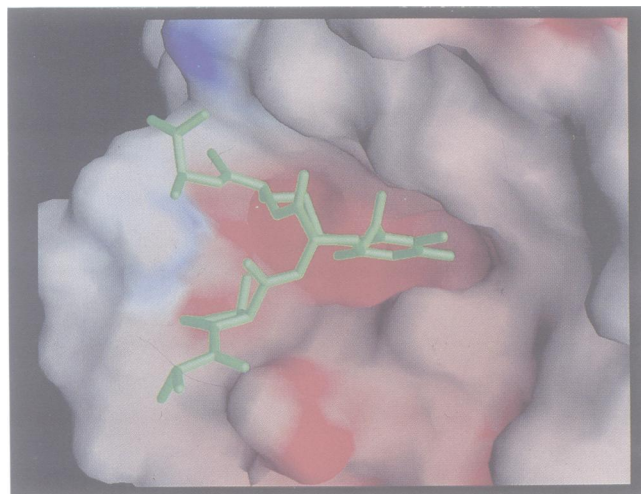
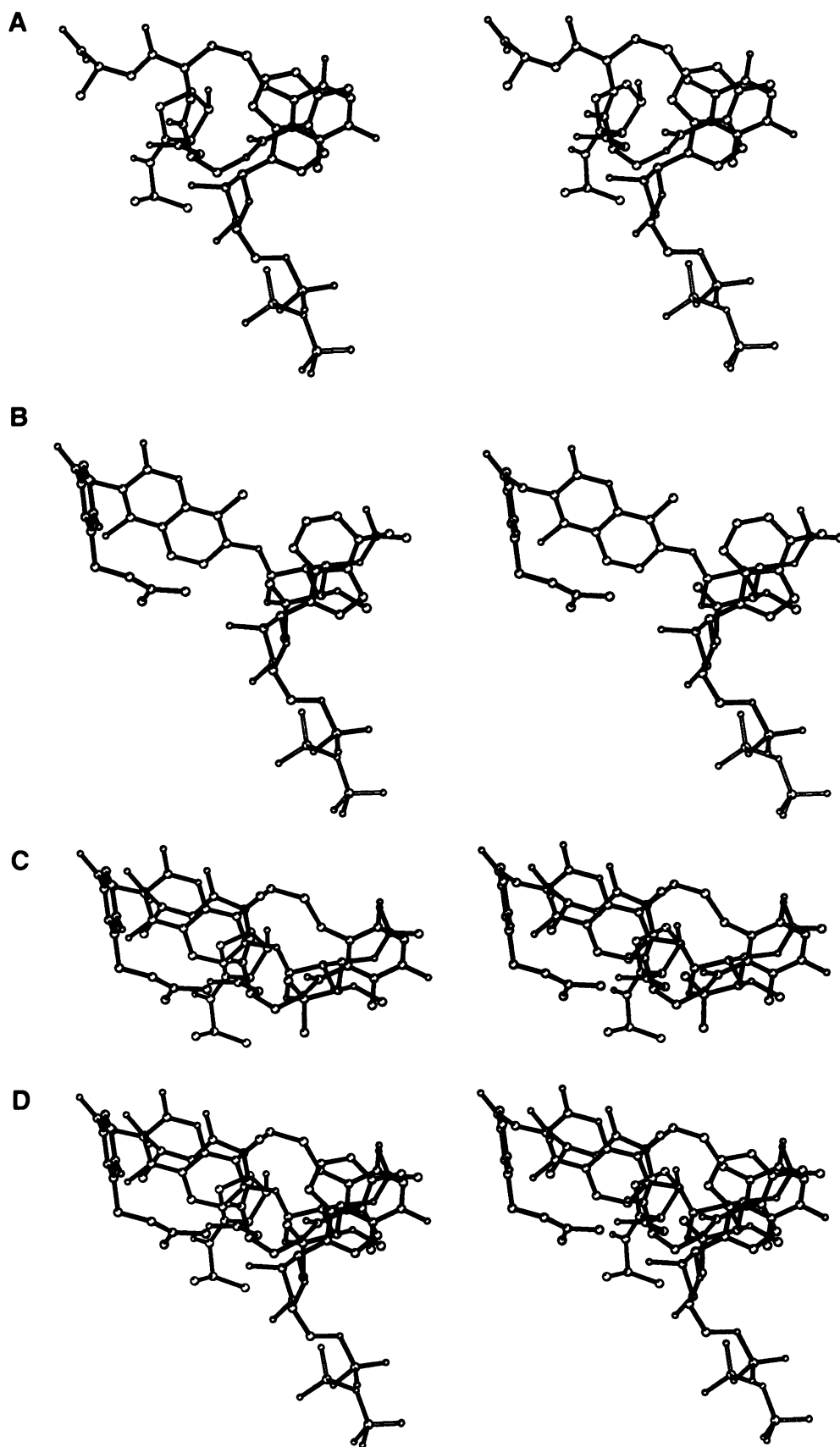


Fig. 5. Complementarity between the protein surface and GR122222X. This figure was prepared using GRASP (Nicholls and Honig, 1991). The red and blue colouration indicates regions of negative and positive potential, respectively.

solution (Klevan and Wang, 1980; Ali *et al.*, 1993). The 43 kDa N-terminal fragment of DNA gyrase B only dimerizes in the presence of ADPNP (Ali *et al.*, 1993). Hence, kinetic studies of these proteins are complicated by the requirement for dimerization of the enzyme for activity (Maxwell, 1993). The rate-limiting step in such kinetic studies becomes the dimerization event, which may not be the case for the intact enzyme complex. The closer proximity of the B proteins in the  $A_2B_2$  complex may explain why their association is apparently more rapid in the intact enzyme. The crystal structure reported here indicates that novobiocin is a classical competitive inhibitor with respect to ATP. This observation is in agreement with the earlier studies of the intact enzyme complex (Sugino *et al.*, 1978).

The *gyrB* genes from three independent coumermycin  $A_1$ -resistant strains of *E.coli* have been sequenced (del Castillo *et al.*, 1991) and all were found to have mutations at Arg136 (to either Leu or Cys). Nine independent spontaneous coumarin-resistant isolates (Contreras and Maxwell, 1992) were found to have mutations at Arg136 (to His, Cys or Ser). The ATPase and supercoiling activities of these three mutants were greatly reduced in comparison with the wild-type enzyme. In the crystal structure of NgyrB–ADPNP, the guanidinium group of Arg136 forms a hydrogen bond with the main chain carbonyl of Tyr5, which is found on the N-terminal arm which wraps around the other subunit to form the dimer (Wigley *et al.*, 1991). The phenolic hydroxyl of Tyr5 forms a hydrogen bond to the 2'-hydroxyl of the ribose of ADPNP (Wigley *et al.*, 1991) and 2'-deoxy ATP is a poor substrate for the ATPase and supercoiling activities of DNA gyrase (Gellert *et al.*, 1976a; Sugino *et al.*, 1978). Loss of the hydrogen bond between Arg136 and Tyr5 in the coumarin-resistant mutants results in a less active enzyme, presumably because of the absence of this important interaction. However, this loss of activity can apparently be tolerated in exchange for the resistance to novobiocin which is conferred by the mutation. Arg136 is also important in a temperature-sensitive coumermycin  $A_1$ -resistant *E.coli*



**Fig. 6.** Stereo diagrams illustrating details of the overlap between the binding sites for: (A) GR122222X and ADPNP, (B) novobiocin and ADPNP, (C) novobiocin and GR122222X, (D) novobiocin, GR122222X and ADPNP. ADPNP is represented with open bonds, novobiocin with shaded bonds and GR122222X with black bonds.

strain (N4177) which has been shown to carry two mutations from wild-type, Arg136Cys and Pro171Ser (Menzel and Gellert, 1983; Contreras and Maxwell, 1992). The drug resistance shown by this strain is probably due to the mutation of Arg136, since the Pro171 mutation is responsible for conferring the temperature-sensitive phenotype. Mutations in *gyrB* from *Borrelia burgdorferi* that confer coumarin resistance have also been identified as changes at Arg133 (Arg136 in the *E.coli* sequence) to Gly or Ile (Samuels *et al.*, 1994).

Other mutations have been described which also confer novobiocin resistance upon DNA gyrase. The *gyrB* gene from a novobiocin-resistant strain of *Haloferax* was sequenced and three differences to wild-type were found at amino acids Gly82, Thr122 and His137 (wild-type protein has Asp82, Ser122 and Arg137; Holmes and Dyall-Smith, 1991). The *E.coli* equivalents are at amino acids Gly81 (which is either glycine or aspartate in *gyrBs*, and mostly valine in topoisomerase IIs), Ser121 (which is totally conserved amongst *gyrBs*, but is lysine in all topoisomerase IIs) and Arg136 (usually arginine in both *gyrBs* and topoisomerase IIs). In combination, these three mutations allowed the novobiocin-resistant strain of *Haloferax* to grow in concentrations of novobiocin some three orders of magnitude higher than the wild-type strain. In the *E.coli* p24–novobiocin complex, Gly81 is situated at the base of a short loop (81–87) that is disordered and in any case is remote from the novobiocin binding site. The second mutation corresponds to Ser121, which is situated at one end of a loop which extends between residues 100 and 120. In the NgyrB–ADPNP complex, this loop forms a ‘lid’ over the ATP binding site, but in the novobiocin complex this loop is flipped up. It is unlikely that a mutation from serine to threonine at position 121 would affect binding of coumarins since this residue is too far from the coumarin binding site, though the possibility of a secondary structural effect resulting from this mutation cannot be ruled out. From the crystal structure of p24–novobiocin one can see that Arg136 forms hydrogen bonds to both the ester and carbonyl oxygens of novobiocin. Mutation to almost any other amino acid would result in the loss of this important interaction. Arg136 is highly conserved amongst gyrases, only *gyrB* from *Spiroplasma citri* and *Streptomyces* do not encode an arginine at this position (Caron and Wang, 1994). Consequently, of the three simultaneous mutations which have been discovered to confer coumarin resistance to DNA gyrase in *Haloferax*, the relative contributions to novobiocin resistance made by these amino acids remain unknown, but it is likely that Arg136 is the predominant mutation.

Another mutation, Gly164Val, confers both temperature sensitivity and chlorobiocin resistance to *E.coli* gyrase (Orr *et al.*, 1979; Contreras and Maxwell, 1992). This mutation does not confer a high degree of coumarin resistance, the IC<sub>50</sub> for coumermycin A<sub>1</sub> is only twice that of the wild-type strain, while for chlorobiocin it is 7-fold higher and for novobiocin 3-fold higher than that of the parental strain (Orr *et al.*, 1979; Contreras and Maxwell, 1992). Gly164 is situated on a strand of  $\beta$ -sheet, but if Gly164 was to be mutated to valine the side chain would point towards the  $\beta$ -strand which includes Asp73. A valine in this position would clash with the carbonyl oxygen of Asp73. This steric hindrance would have to be relieved

in the mutant enzyme and this could affect binding of the coumarins by disrupting the conformation of Asp73, and possibly also Thr165, both of which are important residues for the binding of coumarins.

One of the key residues in binding of ADPNP, novobiocin and GR12222X is Thr165, a totally conserved residue amongst all *gyrBs* and topoisomerase IIs. Thr165 forms a water-mediated hydrogen bond to the adenine ring of ADPNP, therefore it is possible that a mutation at this residue would have an effect on the ATPase and supercoiling activities of DNA gyrase. Mutations at this amino acid might also confer novobiocin resistance because Thr165 forms a water-mediated hydrogen bond with the carbamoyl moiety of novobiocin. Modifications at the carbamoyl of novobiocin result in a drastic reduction in the effectiveness of the drug (Kuo *et al.*, 1991). It is likely that mutation of Thr165 would have a similar effect on the binding of GR12222X, since the hydroxyl at position R<sup>3</sup> of the resorcinol ring forms a water-mediated hydrogen bond with Thr165. Replacement of the hydroxyl with hydrogen at this position reduces the anti-gyrase inhibitory activity by over three orders of magnitude (Goetschi *et al.*, 1993). Therefore, the interaction with Thr165 is of crucial importance, and loss of this interaction would probably result in high levels of resistance to GR12222X.

Novobiocin (novobiocin which lacks the novobiocin moiety) is inactive as a gyrase inhibitor (Reusser and Dolak, 1986). A series of compounds has been made which lack the novobiocin moiety completely or in which it has been replaced by a hydroxyl group (Althaus *et al.*, 1988; Sutcliffe *et al.*, 1992). Interestingly, both groups of compounds are inhibitors of the supercoiling reaction, but the ability to inhibit the ATPase activity requires substitution of the novobiocin by a hydroxyl group. This can be explained by the crystal structures presented here, as the hydroxyl group is sufficient to provide steric hindrance with the N3 atom of the adenine ring of ATP, but the lack of this group could allow the simultaneous binding of both compounds (Figure 6B). In the crystal structure of the 43 kDa B protein fragment complexed with ADPNP, the coumarin binding site is occupied by the N-terminal arm from the adjacent subunit of the dimer. This interaction plays an important role in the proposed mechanism of DNA gyrase (Wigley *et al.*, 1991). Thus, binding of any of the coumarins at this site would prevent supercoiling, yet some complexes would still be able to bind ATP.

One of the problems associated with the clinical application of the coumarin antibiotics is the inhibition of eukaryotic enzymes, such as topoisomerase II (Liu *et al.*, 1980) and DNA polymerase  $\alpha$  (Sung, 1974). It has been shown that the specificity for DNA gyrase over topoisomerase II is much greater for GR12222X than novobiocin (Nakada *et al.*, 1993), making it potentially of greater use as a therapeutic agent. Curiously, many of the specific interactions between all three ligands (GR12222X, ATP and novobiocin) and gyrase are with the same amino acids, notably Asp73 and Thr165, which are highly conserved amongst both gyrases and topoisomerase IIs (Caron and Wang, 1994), suggesting that the reasons for specificity must lie elsewhere. The specificity of GR12222X for gyrase over type II topoisomerases



is presumably conferred by subtle differences between the structures of gyrase and topoisomerase II at the periphery of the ATP binding site. Certainly, GR12222X shows a high degree of complementarity to the molecular surface of the DNA gyrase B subunit (Figure 5).

GR12222X, novobiocin and ADPNP all bind to similar sites in gyrB. The adenine ring of ADPNP overlaps with the resorcinol ring of GR12222X, but they do not lie in the same plane (Figure 6A). The 4'-OMe and 3'-carbamoyl of novobiocin also share this binding site (Figure 6B). One side of the lactone ring of GR12222X runs almost parallel to one side of the sugar and coumarin ring of novobiocin (Figure 6C). The coumarin carbonyl of novobiocin and the carboxyl of the C-terminal alanine of GR12222X, both of which form hydrogen bonds to the guanidium of Arg136, are  $\sim 1.5$  Å apart. In addition, there are a number of ordered water molecules involved in mediating interactions between the three ligands and gyrB. However, the binding sites for the isopentenyl hydroxybenzoate group of novobiocin, the phosphates and part of the ribose of ATP, and the N-terminal two amino acids of GR12222X are discrete and do not overlap (Figure 6D). Attempting to model the binding sites for three such different ligands *ab initio* is a difficult task. The structures presented here demonstrate not only the differences between the binding modes of the ligands but also highlight the important role that bound water molecules play in this process. Information about bound water molecules can only be obtained from high resolution crystal structures, and underlines the need for these data for the design of ligands targeted to particular sites on a protein. The vast binding surface covered by these molecular probes provides a unique system for the design of novel inhibitors of the ATPase activity of DNA gyrase, but also contributes to our understanding of the specificity of protein–ligand interactions in general.

## Materials and methods

### Crystallization and data collection

Crystals of p24 in the presence of both novobiocin and GR12222X were obtained as described previously (Lewis *et al.*, 1994). For the GR12222X complex, crystals belong to the orthorhombic space group  $P2_12_12_1$  with unit cell dimensions of  $a = 68.8$  Å,  $b = 68.6$  Å,  $c = 41.7$  Å and contain one molecule per asymmetric unit. Data were collected from a single crystal using an Enraf-Nonius FAST television area detector with  $\text{CuK}\alpha$  radiation. The measurements were evaluated with the program MADNES (Messerschmidt and Pflugrath, 1987). The CCP4 (CCP4, 1994) programs ROTAVATA and AGROVATA were used to scale and merge the data.

Crystals of the p24–novobiocin complex have unit cell dimensions  $a = 40.3$  Å,  $b = 47.8$  Å,  $c = 111.8$  Å, harbour one molecule per asymmetric unit and are also of the orthorhombic space group  $P2_12_12_1$ . Data were collected from three crystals using a Mar Research imaging plate at beamline 9.5 at the Daresbury Synchrotron Radiation Source, UK, at a wavelength of 0.92 Å. Data were processed with DENZO (Otwinowski, 1993) and scaled and merged with the CCP4 programs ROTAVATA and AGROVATA.

### Structure determination

The structure of the GR12222X complex was solved by molecular replacement using the appropriate protein atom coordinates from the refined structure of the N-terminal 43 kDa DNA gyrase B fragment in a complex with ADPNP, NgyrB–ADPNP (Wigley *et al.*, 1991). The CCP4 programs ALMN and TFEC were used to solve the rotation and translation functions respectively. Using data in the range of 8–3.5 Å, the peak in the rotation search was 5.6 standard deviations (SD) above

**Table 1.** Crystallographic data collection and refinement statistics

Data set	p24–GR12222X	p24–novobiocin
Data collection		
Wavelength (Å)	1.54	0.92
No. of crystals	1	3
Resolution (Å)	2.0	2.7
No. of reflections:		
observed	39 695	14 643
unique	12 940	5931
Completeness (%)	93.7	93.9
$R_{\text{merge}}$ (%)	6.3	9.5
Refinement		
Resolution range (Å)	10.0–2.0	10.0–2.7
$R$ factor (%)	18.4	18.8
No. of reflections	12 246	5465
'Free' $R$ factor (%)	25.6	24.2
No. of reflections	655	324
Total No. of atoms	1711	1690
No. of water molecules	143	96
R.m.s. bond length (Å)	0.013	0.014
R.m.s. bond angle (°)	1.54	2.06
R.m.s. dihedral angles (°)	26.48	26.20
R.m.s. improper angles (°)	1.44	1.66
R.m.s. B all bonds (Å <sup>2</sup> )	2.84	3.20
R.m.s. B all angles (Å <sup>2</sup> )	4.47	5.01
Mean B value, all atoms (Å <sup>2</sup> )	23.0	20.2

the mean. Data between 20 and 3 Å were used for the translation search and the maximal peak was observed at  $>9$  SD above the mean.

The p24–novobiocin complex was also solved by molecular replacement. The coordinates of the protein atoms from the partially refined model of the p24–GR12222X complex were used as the search model. The rotation function was solved with data between 8 and 3.5 Å and yielded a peak of 5.4 SD above the mean. For the translation search, data between 20 and 3 Å were used and a peak was observed of 12 SD above the mean.

### Refinement

In both cases, X-PLOR version 3.1 (Brünger, 1992) was used for refinement procedures. Five percent of each data set was removed from the refinement procedure for use in the free  $R$  value calculation (Brünger, 1993). The solutions from molecular replacement were optimized further by 40 cycles of rigid body refinement using data between 10 and 3.0 Å for the p24–GR12222X complex, and between 10 and 2.7 Å for the p24–novobiocin complex. These models gave  $R$  values (and free  $R$  values in parentheses) of 47.4% (49.9%) and 42.7% (43.5%) respectively. After a round of conjugate gradient positional refinement (60 cycles in total of Powell energy minimization) and simulated annealing molecular dynamics (heating to a temperature of 3000 K, then cooling to 300 K), followed by 25 cycles of restrained individual B factor refinement, the  $R$  value for the p24–GR12222X complex, using data between 10 and 2 Å, was 29.3% (35.3%) and in the case of the p24–novobiocin complex, using data between 10 and 2.7 Å, the  $R$  value was 28.8% (36.6%).

Unit-weighted difference Fourier maps ( $F_o - F_c$  and  $2F_o - F_c$ ) were inspected at this stage. Electron density corresponding to the bound ligands was clear for both structures. Refinement of both complexes was performed by repeated rounds of manual rebuilding with the program FRODO (Jones, 1985), interspersed by conjugate gradient positional refinement (and restrained individual B factor refinement) with the program X-PLOR. At a later stage in refinement, when the  $R$  values had dropped to  $<25\%$ , models for the bound ligands were included in the X-PLOR refinement procedure. At the end of the refinement procedure, the  $R$  factor was 18.4% (25.6%) for the p24–GR12222X complex and 18.8% (24.2%) for the p24–novobiocin complex.

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## References

- Ali, J.A., Jackson, A.P., Howells, A.J. and Maxwell, A. (1993) The 43 kDa N-terminal fragment of the gyrase B protein hydrolyses ATP and binds coumarin drugs. *Biochemistry*, **32**, 2717–2724.
- Althaus, I.W., Dolak, L. and Reusser, F. (1988) Coumarins as inhibitors of bacterial DNA gyrase. *J. Antibiot.*, **41**, 373–376.
- Brünger, A.T. (1992) *X-PLOR, Version 3.1: A System for X-ray Crystallography and NMR*. Yale University Press, New Haven, CT.
- Brünger, A.T. (1993) Assessment of phase accuracy by cross validation—the free R-value—methods and applications. *Acta Crystallogr.*, **D49**, 24–36.
- Caron, P. and Wang, J.C. (1994) Alignment of primary sequences of DNA topoisomerases. *Adv. Pharmacol.*, **29A**, 271–297.
- Carson, M. (1991) Ribbons 2.0. *J. Appl. Crystallogr.*, **24**, 958–961.
- CCP4 (1994) CCP4 suite: programs for protein crystallography. *Acta Crystallogr.*, **D50**, 760–763.
- Celia, H., Hoermann, L., Schiltz, P., Lebeau, L., Mallouh, V., Wigley, D.B., Wang, J.C., Mioskowski, C. and Oudet, P. (1994) Three-dimensional model of *Escherichia coli* gyrase-B subunit crystallised in 2-dimensions on novobiocin-linked phospholipid films. *J. Mol. Biol.*, **236**, 618–628.
- Contreras, A. and Maxwell, A. (1992) GyrB mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase. *Mol. Microbiol.*, **6**, 1617–1624.
- del Castillo, I., Vizán, J.L., Rodríguez-Saínz, M.C. and Moreno, F. (1991) An unusual mechanism for resistance to the antibiotic coumermycin A<sub>1</sub>. *Proc. Natl Acad. Sci. USA*, **88**, 8860–8864.
- Fersht, A.R. (1985) *Enzyme Structure and Mechanism*. W.H. Freeman and Company, New York.
- Gellert, M., Mizuuchi, K., O'Dea, M.H. and Nash, H.A. (1976a) DNA gyrase: an enzyme that introduces superhelical turns into DNA. *Proc. Natl Acad. Sci. USA*, **73**, 3872–3876.
- Gellert, M., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1976b) Novobiocin and coumermycin inhibit DNA supercoiling catalysed by DNA gyrase. *Proc. Natl Acad. Sci. USA*, **73**, 4474–4478.
- Gilbert, E.J. and Maxwell, A. (1994) The 24 kDa N-terminal sub-domain of the DNA gyrase B protein binds coumarin drugs. *Mol. Microbiol.*, **12**, 365–373.
- Goetschi, E., Angehrn, P., Gmuender, H., Hebeisen, P., Link, H., Masciadri, R. and Nielsen, J. (1993) Cyclothalidone and its congeners: a new class of DNA gyrase inhibitors. *Pharmacol. Ther.*, **60**, 367–380.
- Hoeksema, H., Johnson, J.L. and Hinman, J.W. (1955) Structural studies on streptomycin, a new antibiotic. *J. Am. Chem. Soc.*, **78**, 6710–6711.
- Holmes, M.L. and Dyall-Smith, M.L. (1991) Mutations in DNA gyrase result in novobiocin resistance in halophilic archaeobacteria. *J. Bacteriol.*, **173**, 642–648.
- Jones, T.A. (1985) Interactive computer graphics: FRODO. *Methods Enzymol.*, **115**, 157–171.
- Kirchhausen, T., Wang, J.C. and Harrison, S.C. (1985) DNA gyrase and its complexes with DNA: direct observation by electron microscopy. *Cell*, **41**, 933–943.
- Klevan, L. and Wang, J.C. (1980) A DNA–DNA gyrase complex containing 140 base pairs of DNA and an  $\alpha_2\beta_2$  protein core. *Biochemistry*, **19**, 5229–5234.
- Krueger, S., Zaccari, G., Wlodawer, A., Langowski, J., O'Dea, M., Maxwell, A. and Gellert, M. (1990) Neutron and light-scattering studies of DNA gyrase and its complex with DNA. *J. Mol. Biol.*, **211**, 211–220.
- Kuo, M.S., Yurek, D.A., Chirby, D.G., Cialdella, J.I. and Marshall, V.P. (1991) Microbial O-carbamoylation of novobiocin. *J. Antibiot.*, **44**, 1096–1100.
- Lebeau, L., Regnier, E., Schultz, P., Wang, J.C., Mioskowski, C. and Oudet, P. (1990) Two-dimensional crystallisation of DNA gyrase B subunit on specifically designed lipid monolayers. *FEBS Lett.*, **267**, 38–42.
- Lewis, R.J., Singh, O.M.P., Smith, C.V., Maxwell, A., Skarzynski, T., Wonacott, A.J. and Wigley, D.B. (1994) Crystallisation of inhibitor complexes of an N-terminal 24 kDa fragment of the DNA gyrase B protein. *J. Mol. Biol.*, **241**, 128–130.
- Liu, L.F., Liu, C.-C. and Alberts, B.M. (1980) Type II DNA topoisomerases: enzymes that can unknot topologically knotted DNA molecule via a reversible double-stranded break. *Cell*, **19**, 697–708.
- Maxwell, A. (1993) The interaction between coumarin drugs and DNA gyrase. *Mol. Microbiol.*, **9**, 681–686.
- Maxwell, A. and Gellert, M. (1984) The DNA dependence of the ATPase activity of DNA gyrase. *J. Biol. Chem.*, **259**, 14472–14480.
- Menzel, R. and Gellert, M. (1983) Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. *Cell*, **34**, 105–113.
- Messerschmidt, A. and Pflugrath, J.W. (1987) Crystal orientation and X-ray pattern prediction routines for area-detector diffractometer systems in macromolecular crystallography. *J. Appl. Crystallogr.*, **20**, 306–315.
- Mizuuchi, K., O'Dea, M.H. and Gellert, M. (1978) DNA gyrase: subunit structure and ATPase activity of the purified enzyme. *Proc. Natl Acad. Sci. USA*, **75**, 5960–5963.
- Nakada, N., Shimada, H., Hirata, T., Aoki, Y., Kamiyama, T., Watanabe, J. and Arisawa, M. (1993) Biological characterisation of cyclothalidone, a new DNA gyrase inhibitor. *Antimicrob. Agents Chemother.*, **37**, 2656–2661.
- Nakada, N., Gmuender, H., Hirata, T. and Arisawa, M. (1994) Mechanism of inhibition of DNA gyrase by cyclothalidone, a novel DNA gyrase inhibitor. *Antimicrob. Agents Chemother.*, **38**, 1966–1973.
- Nicholls, A. and Honig, B. (1991) A rapid finite difference algorithm, utilising successive over relaxation to solve the Poisson–Boltzmann equation. *J. Comput. Chem.*, **12**, 435–445.
- Orr, L., Fairweather, N.F., Holland, I.B. and Pritchard, R.H. (1979) Isolation and characterisation of a strain carrying a conditional lethal mutation in the *cou* gene of *Escherichia coli* K12. *Mol. Gen. Genet.*, **177**, 103–112.
- Otwinski, Z. (1993) *Data Collection and Processing*. SERC, Daresbury DL/SC1/R34, pp. 56–62.
- Rau, D.C., Gellert, M., Thoma, F. and Maxwell, A. (1987) Structure of the DNA gyrase–DNA complex as revealed by transient electric dichroism. *J. Mol. Biol.*, **193**, 555–569.
- Reece, R.J. and Maxwell, A. (1991a) DNA gyrase: structure and function. *Crit. Rev. Biochem. Mol. Biol.*, **26**, 335–375.
- Reece, R.J. and Maxwell, A. (1991b) Probing the limits of the DNA breakage–reunion domain of the *Escherichia coli* DNA gyrase A protein. *J. Biol. Chem.*, **266**, 3540–3546.
- Reece, R.J. and Maxwell, A. (1991c) The C-terminal domain of the *Escherichia coli* DNA gyrase A subunit is a DNA-binding protein. *Nucleic Acids Res.*, **19**, 1399–1405.
- Reusser, F. and Dolak, L. (1986) Novenamine is the active moiety in novobiocin. *J. Antibiot.*, **39**, 272–274.
- Samuels, D.S., Marconi, R.T., Huang, W.M. and Garon, C.F. (1994) *gyrB* mutations in coumermycin A<sub>1</sub>-resistant *Borrelia burgdorferi*. *J. Bacteriol.*, **176**, 3072–3075.
- Staudenbauer, W.L. and Orr, E. (1981) DNA gyrase: affinity chromatography on novobiocin–Sepharose and catalytic properties. *Nucleic Acids Res.*, **9**, 3589–3603.
- Sugino, A. and Cozzarelli, N.R. (1980) The intrinsic ATPase of DNA gyrase. *J. Biol. Chem.*, **255**, 6299–6306.
- Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L. and Cozzarelli, N.R. (1978) Energy coupling in DNA gyrase and the mechanism of action of novobiocin. *Proc. Natl Acad. Sci. USA*, **75**, 4838–4842.
- Sung, S.C. (1974) Effect of novobiocin on DNA-dependent DNA polymerases from developing rat brain. *Biochim. Biophys. Acta*, **361**, 115–120.
- Sutcliffe, J., Yang, B., Anderson, M., Goldsmith, M., Norcia, M., O'Rourke, D. and Wondrack, L. (1992) 3-Carbamoyl-4-hydroxycoumarin compounds interact with DNA gyrase in a novel fashion. *Proceedings of the Fourth Conference on DNA Topoisomerases in Therapy*. Abstract 22, p. 29.
- Tamura, J.K. and Gellert, M. (1990) Characterisation of the ATP binding site on *Escherichia coli* DNA gyrase. Affinity labelling of lys103 and lys110 of the B subunit by pyridoxal 5'-diphospho-5'-adenosine. *J. Biol. Chem.*, **265**, 21342–21349.
- Tamura, J.K., Bates, A.D. and Gellert, M. (1992) Slow binding of 5'-adenyl- $\beta$ - $\gamma$ -imidodiphosphate to *Escherichia coli* DNA gyrase: evidence for cooperativity in nucleotide binding. *J. Biol. Chem.*, **267**, 9214–9222.
- Wang, J.C. (1985) DNA topoisomerases. *Annu. Rev. Biochem.*, **54**, 665–697.
- Wigley, D.B., Davies, G.J., Dodson, E.J., Maxwell, A. and Dodson, G. (1991) Crystal structure of the N-terminal domain of the DNA gyrase B protein. *Nature*, **351**, 624–629.

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